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## Identification and characterization of novel catalytic bioscavengers of organophosphorus nerve agents

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### ABSTRACT

In an effort to discover novel catalytic bioscavengers of organophosphorus (OP) nerve agents, cell lysates from a diverse set of bacterial strains were screened for their capacity to hydrolyze the OP nerve agents VX, VR, and soman (GD). The library of bacterial strains was identified using both random and rational approaches. Specifically, two representative strains from eight categories of extremophiles were chosen at random. For the rational approach, the protein sequence of organophosphorus hydrolase (OPH) from *Brevundimonas diminuta* was searched against a non-redundant protein database using the Basic Local Alignment Search Tool to find regions of local similarity between sequences. Over 15 protein sequences with significant sequence similarity to OPH were identified from a variety of bacterial strains. Some of these matches were based on predicted protein structures derived from bacterial genome sequences rather than from *bona fide* proteins isolated from bacteria. Of the 25 strains selected for nerve agent testing, three bacterial strains had measurable levels of OP hydrolase activity. These strains are *Ammoniphilus oxalaticus*, *Haloarcula* sp., and *Micromonospora aurantiaca*. Lysates from *A. oxalaticus* had detectable hydrolysis of VR; *Haloarcula* sp. had appreciable hydrolysis of VX and VR, whereas lysates from *M. aurantiaca* had detectable hydrolysis of VR and GD.

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### 1. Introduction

Organophosphorus (OP) compounds are highly toxic substances that have been used as chemical warfare agents [1–3]. OP nerve agents readily bind covalently to the active site serine in acetylcholinesterase (AChE), thereby inhibiting the ability of AChE to terminate cholinergic neural transmissions [4]. Several drugs can be administered post-exposure that attempt to either reactivate AChE or reverse the toxic symptoms of nerve agent poisoning (reviewed in [5]). The cholinergic blocker atropine reduces binding of acetylcholine at muscarinic receptors, whereas 2-pralidoxime chloride, an oxime nucleophile, reactivates AChE by displacing the phosphoryl group left on the active site serine after nerve agent inhibition. The benzodiazepine diazepam is administered to control tremors

and convulsions. Individuals at high risk for exposure to soman can be pretreated with the spontaneously reactivating AChE inhibitor pyridostigmine bromide, which transiently obscures the active site of a fraction of peripheral AChE molecules, thereby protecting the enzyme from irreversible inhibition by nerve agent [5]. While the current treatment regimen is effective in averting lethality if administered soon after nerve agent intoxication, it does not prevent performance deficits, loss of consciousness, or potential brain damage. Current research efforts are focused on the development of catalytic bioscavengers to be administered as prophylactics. These enzymes would circulate in the blood stream and hydrolyze OP compounds, thus removing the nerve agents from circulation before they can reach their physiological target. Paraoxonase-1 [6] and organophosphorus hydrolase (OPH; [7]) from *Brevundimonas diminuta* are two leading candidate catalytic bioscavenger enzyme platforms under development. Although variants on each platform have been identified that catalyze the rapid hydrolysis of G-agents, an effective scavenger of V-agents has not yet been discovered. To date, a comprehensive screen of multiple strains

Abbreviations: OP, organophosphorus; OPH, organophosphorus hydrolase; AChE, acetylcholinesterase; Px, paraoxon.

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of bacteria for enzymes with the capacity to hydrolyze *bona fide* nerve agents has not been reported. This paper describes the selection and screening of a diverse set of bacterial strains for anti-nerve agent activity. Our initial results indicate that *Ammoniphilus oxalaticus*, *Haloarcula* sp., and *Micromonospora aurantiaca* contain previously unidentified OP hydrolase activities.

## 2. Materials and methods

### 2.1. Bacterial strains

All bacterial strains were purchased from American Type Culture Collection (Manassas, VA). Each strain was grown according to the manufacturer's specifications without modifications (details of growth conditions are available at <http://www.atcc.org>) to an OD<sub>600</sub> of 0.8. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C. The resulting cell pellets were resuspended in cold phosphate buffered saline (PBS), pH 7.4, and lysed by sonication on ice. No protease inhibitors were added to these lysates. Any insoluble protein and cell debris were removed by centrifugation at 12,000 rpm for 20 min at 4 °C. The soluble cell lysate fraction was kept at 4 °C if OP hydrolase testing was conducted on the same day. Otherwise, samples were stored at –80 °C for future OP hydrolase testing.

### 2.2. V-agent hydrolysis assays

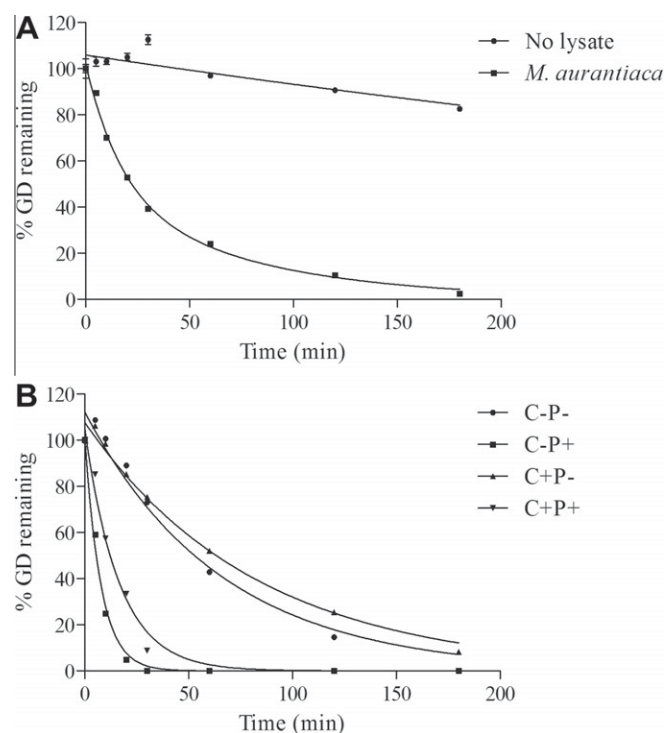
Racemic VX (*O*-ethyl *S*-(2-diisopropylaminoethyl) methylphosphonothiolate and VR (*O*-isobutyl *S*-(2-diethylaminoethyl) methylphosphonothiolate were obtained from the U.S. Army Edgewood Chemical Biological Center (ECBC, Aberdeen Proving Ground, MD). Stock solutions of each V-agent were prepared at 0.9 mg/mL in saline and stored at –80 °C. A modified Ellman-based colorimetric assay was used to measure hydrolysis of VX and VR [8]. Briefly, 130 µL cell lysate was incubated with a final concentration of 1.4 mM V-agent in the presence of 5 mM 5,5'-dithiobis(2-nitrobenzoic acid) in a 96-well microplate. Final volume for each well was 240 µL. Hydrolysis of V-agent was monitored at A<sub>412</sub> ( $\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$ ) for 20 min at room temperature. Each lysate was tested in triplicate.

### 2.3. Soman hydrolysis assay

Racemic soman (GD) was obtained from ECBC. Stock solutions of GD in saline were prepared at 2 mg/mL and stored at –80 °C. For the assay, 700 µL cell lysate or PBS (negative control) was incubated with a final concentration of 0.5 mM GD and a total volume of 900 µL. At various time intervals, 100 µL aliquots were removed and extracted with an equal volume of ethyl acetate containing 50 µM diisopropyl fluorophosphate (added as an internal standard). The extraction inactivates any enzymatic activity and prevents racemization of non-hydrolyzed GD stereoisomers. The organic layer containing non-hydrolyzed GD was removed, dried over molecular sieve beads, and analyzed by gas chromatography/mass spectrometry using a modification (Kajih et al. in preparation) of previously described methods [9]. Each lysate was examined in triplicate. The data shown in Fig. 1A were fitted to a two-phase exponential decay, and the data in Fig. 1B were fitted to a one-phase exponential decay using Prism 5.04 (GraphPad Software, Inc.) with the plateau constraint set to zero.

### 2.4. Scanning electron microscopy

Pelleted samples were fixed using buffered 1.6% paraformaldehyde/2.5% glutaraldehyde. Following fixation, samples were osmi-



**Fig. 1.** Hydrolysis of racemic GD in bacterial cell lysates prepared from *M. aurantiaca*. Clarified cell lysates from *M. aurantiaca* were incubated with 0.5 mM racemic GD. Aliquots were removed at various time points and extracted with an equal volume of ethyl acetate. The amount of GD remaining in each sample was determined by GC/MS analysis. Analysis of total GD (A) and each of the four stereoisomers (B) remaining at each time point is shown  $\pm$  standard deviation ( $<5\%$ ). “No lysate” represents spontaneous hydrolysis of GD in the absence of any bacterial lysate.

cated and processed by standard dehydration protocol to critical point drying. Dried samples were ion beam coated with gold/palladium and imaged using a JEOL JSM-7401F field emission scanning electron microscope.

### 2.5. Enrichment of OP hydrolase protein(s) from *M. aurantiaca*

The soluble cell lysate prepared from *M. aurantiaca* was fractionated with increasing concentrations (20%, 40%, 60%, and 80%) of ammonium sulfate. The precipitated proteins were removed at each step by centrifugation at 14,000 rpm for 20 min, and the protein pellets were resuspended in PBS. After residual ammonium sulfate was removed by dialysis, the extracted protein solutions were tested for activity against the substrates VR and paraoxon. Enzymatic activity against each substrate was found in the 40–60% ammonium sulfate fraction. This fraction was run over a DEAE Sepharose Fast Flow anion exchange column (GE Healthcare Life Sciences, Pittsburgh, PA) equilibrated with 10 mM KPO<sub>4</sub>, 20 mM KCl, pH 7.4. Any protein remaining bound to the ion exchange group was eluted from the column with an increasing KCl gradient (0.02 M to 1.0 M). Collected fractions were assayed for activities against paraoxon and VR.

### 2.6. Paraoxon hydrolysis assay

One-hundred microliters lysate samples from *M. aurantiaca* enriched for OP hydrolase activity were incubated in the presence of 8 mM paraoxon (Sigma–Aldrich, St. Louis, MO) in PBS. The final volume in the well was 200 µL. Formation of *p*-nitrophenol was followed in a 96-well microplate at A<sub>412</sub> ( $\epsilon = 17,000 \text{ M}^{-1} \text{ cm}^{-1}$ )

for 20 min at room temperature. Samples were assayed in duplicate.

### 3. Results and discussion

#### 3.1. Selection of bacterial strains for OP hydrolase testing

The amino acid sequence of organophosphorus hydrolase (OPH) from *B. diminuta* was searched against a non-redundant protein database using Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov>) to find regions of local similarity between sequences. Bacterial strains corresponding to the top ten candidate matches from the BLAST search were chosen to be examined for OP hydrolase activity. These strains contained protein sequences that ranged from 28–32% identity and 46–51% similarity to the OPH amino acid sequence. Only two of the selected strains (*Sulfolobus solfataricus* P2 and *Deinococcus radiodurans*) had amino acid sequence alignments from *bona fide* bacterial proteins, with the rest constituting open reading frames or predicted protein products. As an additional approach to the rational selection of bacterial strains described above, two representative strains from the following categories were selected for analysis: acidophile, alkaliphile, archaea, halophile, methylotroph, phototroph, sulfur oxidizer, and sulfate reducer. The bacterial strains chosen for analysis, as well as the selection method used, are listed in Table 1. Each strain was grown under optimal media and temperature conditions, and clarified cell lysates were prepared.

#### 3.2. OP hydrolase screening

Each of the clarified cell lysates was examined for the ability to hydrolyze the V-agents VX and VR using a modified Ellman assay. Only lysates from three bacterial strains had detectable levels of V-agent hydrolysis (Table 2). *Haloarcula* sp. was able to hydrolyze both VX and VR, whereas hydrolysis of VR but not VX was observed in lysates prepared from *Ammoniphilus oxalaticus* (*A. oxalaticus*) and *Micromonospora aurantiaca* (*M. aurantiaca*). *M. aurantiaca* cells are orange in color when grown in liquid culture. As a result, cell lysates prepared from this strain are also orange, which resulted in a high background level in the V-agent assay. Therefore, the

**Table 1**  
Bacterial strains selected for OP hydrolase screening.

Bacterial strain	Selection basis
<i>Acidithiobacillus ferrooxidans</i>	Acidophile
<i>Acidomonas methanolica</i>	Acidophile/methylotroph
<i>Aeromonas jandaei</i>	BLAST
<i>Ammoniphilus oxalaticus</i>	Alkaliphile
<i>Atopobium rimae</i>	BLAST
<i>Bacillus halophilus</i>	Halophile
<i>Beggiatoa alba</i>	Sulfur oxidizer
<i>Brevibacterium linens</i>	BLAST
<i>Deinococcus radiodurans</i>	BLAST
<i>Desulfotobacterium dehalogenans</i>	Sulfate reducer
<i>Geobacillus thermodenitrificans</i>	BLAST
<i>Haloarcula</i> sp.	Archaea
<i>Halotheobacillus hydrothermalis</i>	Sulfur oxidizer
<i>Halotheobacillus neapolitanus</i>	Halophile
<i>Hyphomicrobium methylovorum</i>	Methylotroph
<i>Micromonospora aurantiaca</i>	BLAST
<i>Natrialba asiatica</i>	Archaea
<i>Paracoccus alcaliphilus</i>	Alkaliphile
<i>Rhodobacter capsulatus</i>	Phototroph
<i>Rhodopseudomonas palustris</i>	Phototroph
<i>Shewanella algae</i>	Sulfate reducer
<i>Streptoparvum roseum</i>	BLAST
<i>Sulfolobus solfataricus</i> P2	BLAST
<i>Thermomonospora curvata</i>	BLAST
<i>Tsukamurella paurometabola</i>	BLAST

**Table 2**  
Bacterial strains with detectable V-agent hydrolysis.

Bacterial strain	VX (pmol/min)	VR (pmol/min)
<i>Ammoniphilus oxalaticus</i>	ND	120 ± 3
<i>Haloarcula</i> sp.	40 ± 3	110 ± 8
<i>Micromonospora aurantiaca</i>	ND	170 ± 5*

ND – none detected.

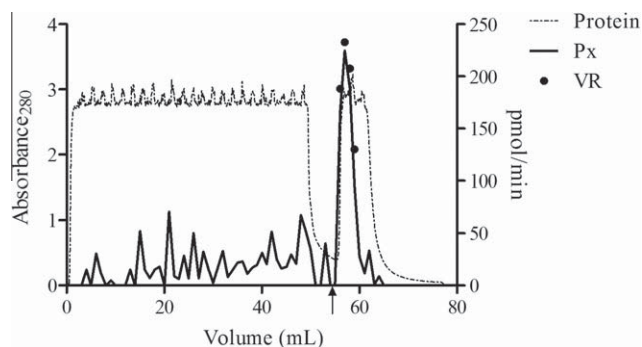
\* Cell lysate was diluted 1:10 prior to assay.

*M. aurantiaca* lysate was diluted sufficiently such that V-agent hydrolysis could be assessed. Using the working assumption that data obtained from this assay will be linear with respect to lysate concentration in the ranges utilized here, we determined that the VR hydrolysis in lysates prepared from *M. aurantiaca* could be as high as 1.7 nmol/min. Although the values presented in Table 2 are relatively low, these reduced activity levels could result from either lower intrinsic activity of candidate enzyme(s) or low expression levels of the enzyme(s) in the lysates.

Clarified cell lysates prepared from each of the 25 bacterial strains were also tested for the capacity to hydrolyze soman (GD). Only lysates from *M. aurantiaca* displayed any detectable hydrolysis of GD, with apparent  $k_{\text{fast}}$  ( $0.0585 \pm 0.0241 \text{ min}^{-1}$ ) and  $k_{\text{slow}}$  ( $0.0133 \pm 0.0053 \text{ min}^{-1}$ ) rates (Fig. 1A). The spontaneous hydrolysis of GD is represented by the “no lysate” control ( $k = 0.0013 \pm 0.0003 \text{ min}^{-1}$ ). GD contains both a chiral phosphorus atom and a chiral carbon atom and therefore exists as four stereoisomers (C+P+, C+P–, C–P+, C–P–). The C ± P-isomers are more toxic *in vivo* and more readily inhibit AChE *in vitro* than the P+ isomers [10,11]. The activity associated with cell lysates prepared from *M. aurantiaca* hydrolyzed GD in a stereoselective manner with modest preference against the less toxic P+ isomers (Fig. 1B), with apparent  $k$  values of  $0.0608 \pm 0.0060$ ,  $0.0121 \pm 0.0009$ ,  $0.1285 \pm 0.0083$ , and  $0.0153 \pm 0.0019 \text{ min}^{-1}$ , for isomers C+P+, C+P–, C–P+, C–P–, respectively. These results are in reasonable agreement with the rates determined from Fig. 1A, where  $k_{\text{fast}}$  likely reflects hydrolysis of the C ± P+ isomers and  $k_{\text{slow}}$  represents hydrolysis of the C ± P– isomers. Because lysates from *M. aurantiaca* had the capacity to hydrolyze both VR and GD, subsequent efforts were focused on identifying the enzyme(s) responsible for the observed OP hydrolase activity in this strain.

#### 3.3. Enrichment of OP hydrolase activity in *M. aurantiaca* lysates

Ammonium sulfate precipitation was used as the first step in the purification of the protein(s) responsible for the OP hydrolase



**Fig. 2.** Enrichment of OP hydrolase protein(s) from *M. aurantiaca*. Proteins precipitated with 40–60% ammonium sulfate were loaded on a DEAE Sepharose column. Any proteins remaining bound to the ion exchange resin were eluted with increasing concentrations of KCl. UV detection (left y-axis) of proteins either flowing through (to the left of the arrow) or eluting from (to the right of the arrow) the column is depicted by the dashed line. The black arrow on the x-axis indicates the initiation of the KCl gradient. Hydrolysis of paraoxon (Px; solid line) and VR (solid circles) are shown (right y-axis).



activity in lysates from *M. aurantiaca*. Hydrolysis of VR was observed in the 40–60% ammonium sulfate fraction. Interestingly, this fraction also had the capacity to hydrolyze paraoxon (the toxic metabolite of the pesticide parathion). The 40–60% ammonium sulfate fraction was run on a DEAE Sepharose anion exchange column, and any proteins remaining bound to the resin were eluted from the column with increasing concentrations of KCl. The chromatogram depicting the UV detection of proteins either flowing through or eluting from the column is shown in Fig. 2 (dashed line). Each fraction was tested for the capacity to hydrolyze paraoxon. The results demonstrate that the protein(s) responsible for this activity bound to the column and was eluted with KCl in four fractions (Fig. 2; solid line). Each of these fractions was also able to hydrolyze VR (Fig. 2; solid circles). It is possible that two or more

unique proteins are responsible for these enzymatic activities against paraoxon and VR. The capacity of these fractions to catalyze the hydrolysis of GD has not yet been examined. Methods to improve the purification of the OP hydrolase protein(s) from *M. aurantiaca* are being pursued in an effort to identify the enzyme(s) responsible.

### 3.4. Microscopy of *M. aurantiaca*

*M. aurantiaca* is an aerobic, gram-positive filamentous bacteria normally found in soil. This strain has the capability to form spores and branched mycelia. *M. aurantiaca* grow as small orange spheres in culture (Fig. 3A). When analyzed by the Brown and Brenn Gram stain, gram-positive bacteria were observed, as expected (Fig. 3B, black arrows). An orange sphere was bisected and inspected by scanning electron microscopy to reveal a mass of hyphae (Fig. 3C).

## 4. Conclusions

Catalytic bioscavengers, administered prior to nerve agent exposure, have the potential to minimize or eliminate the need for conventional therapeutic treatments since the enzymes have the capacity to hydrolyze OP compounds before the compounds can exit the blood stream and inhibit AChE. Although several promising catalytic scavengers of G-agents have been engineered, a V-agent scavenger with sufficient activity to afford *in vivo* protection has not been identified to date. In this paper, we describe screening for novel bacterial OP hydrolases based on homology to OPH from *B. diminuta*. This screen could have also been performed with other OP hydrolases such as paraoxonase-1 [6,8] and prolidase [12]. Any bacterial protein identified as a candidate therapeutic drug would likely require modifications to minimize any immune response that might lead to rapid clearance from circulation.

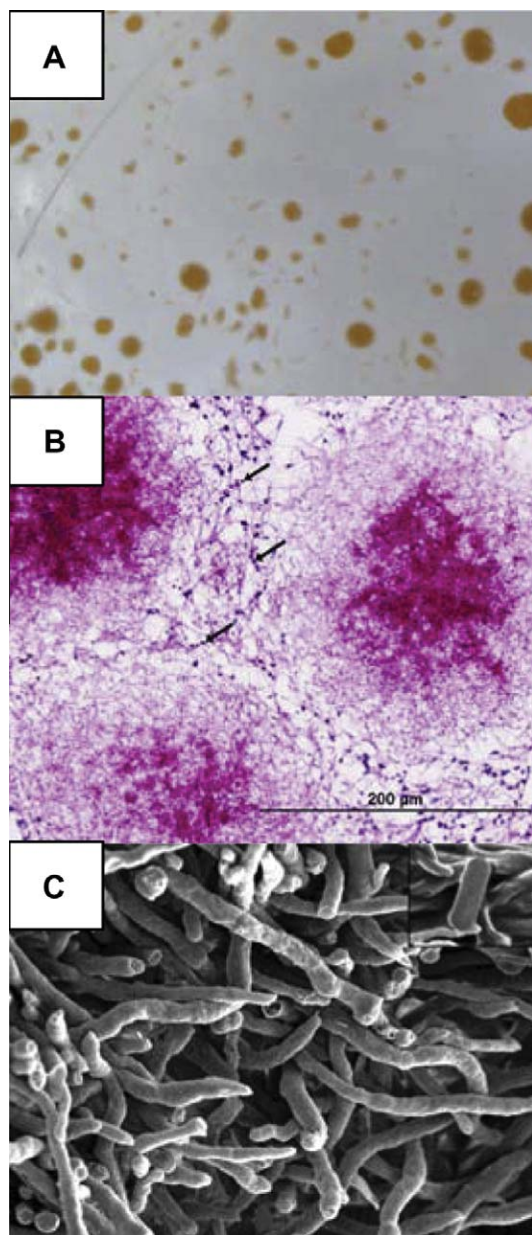
Out of 25 bacterial strains tested in this study, we identified three strains (*A. oxalaticus*, *Haloarcula* sp., and *M. aurantiaca*) with activities against VX, VR, and/or GD. Interestingly, two of these strains (*A. oxalaticus* and *Haloarcula* sp.) were chosen randomly. We recognize the possibility that our screen may have failed to identify bacterial strains in Table 1 with OP hydrolase activity, if that activity was associated with low abundance protein(s). Since the strain *M. aurantiaca* was selected from the BLAST search, the amino acid sequence that aligned with OPH will be cloned and expressed in *Escherichia coli* to determine if the OP hydrolase activity is associated with this gene product. Efforts have also been focused on independent identification of the enzyme by purification of the protein directly from *M. aurantiaca* as it is possible that the observed activities result from more than one enzyme. Once identified, this novel enzyme(s) could function as an OP hydrolase platform for the design and expression of novel catalytic bioscavengers of both G- and V-agents.

## Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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**Fig. 3.** Microscopy of orange spheres from *M. aurantiaca*. (A) Light micrograph of an actively growing liquid culture from *M. aurantiaca* under 10× magnification. (B) Brown and Brenn Gram stain for gram-positive bacteria. Black arrows point to bacteria positive for the gram stain. (C) Scanning electron micrograph from the core of a bisected sphere. The inset is of a bacteria rod from the periphery of the sphere.

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